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Defective autophagy in vascular smooth muscle cells alters contractility and Ca\(^{2+}\) homeostasis in mice

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Submitted 11 September 2014; accepted in final form 6 January 2015

Michiels CF, Fransen P, De Munck DG, De Meyer GR, Martinet W. Defective autophagy in vascular smooth muscle cells alters contractility and Ca\(^{2+}\) homeostasis in mice. Am J Physiol Heart Circ Physiol 308: H557–H567, 2015. First published January 9, 2015; doi:10.1152/ajpheart.00659.2014.—Autophagy is an evolutionary preserved process that prevents the accumulation of unwanted cytosolic material through the formation of autophagosomes. Although autophagy has been extensively studied to understand its function in normal physiology, the role of vascular smooth muscle (SM) cell (VSMC) autophagy in Ca\(^{2+}\) mobilization and contraction remains poorly understood. Recent evidence shows that autophagy is involved in controlling contractile function and Ca\(^{2+}\) homeostasis in certain cell types. Therefore, autophagy might also regulate contractile capacity and Ca\(^{2+}\)-mobilizing pathways in VSMCs. Contractility (organ chambers) and Ca\(^{2+}\) homeostasis (myograph) were investigated in aortic segments of 3.5-mo-old mice containing a SM cell-specific deletion of autophagy-related 7 (Atg7fl/Atg7fl SM22α-Cre\(^{−}\)mice) and in segments of corresponding control control mice (Atg7\(^{+/+}\) SM22α-Cre\(^{+}\)). Our results indicate that voltage-gated Ca\(^{2+}\) channels (VGCCs) of Atg7\(^{+/+}\) SM22α-Cre\(^{−}\) VSMCs were more sensitive to depolarization, independent of changes in resting membrane potential. Contractions elicited with K\(^{+}\) (50 mM) or the VGCC agonist BAY K8644 (100 mM) were significantly higher due to increased VGCC expression and activity. Interestingly, the sarcoplasmic reticulum of Atg7\(^{+/+}\) SM22α-Cre\(^{−}\) VSMCs was enlarged, which, combined with increased sarco(endo)plasmic reticulum Ca\(^{2+}\) ATPase 2 expression and higher store-operated Ca\(^{2+}\) entry, promoted inositol 1,4,5-trisphosphate-mediated contractions of Atg7\(^{+/+}\) SM22α-Cre\(^{−}\) segments and maximized the Ca\(^{2+}\) storing capacity of the sarcoplasmic reticulum. Moreover, decreased plasma membrane Ca\(^{2+}\)-ATPase expression in Atg7\(^{+/+}\) SM22α-Cre\(^{−}\) VSMCs hampered Ca\(^{2+}\) extrusion to the extracellular environment. Overall, our study indicates that defective autophagy in VSMCs leads to an imbalance between Ca\(^{2+}\) release/influx and Ca\(^{2+}\) reuptake/extrusion, resulting in higher basal Ca\(^{2+}\) concentrations and significant effects on vascular reactivity.

Autophagy: vascular smooth muscle cells; contractility; calcium homeostasis; sarcoplasmic reticulum

Autophagy is a basally active subcellular housekeeping mechanism that protects the cell against the accumulation of misfolded or damaged organelles and protein aggregates (35). Generally, there are three types of autophagy: 1) microautophagy, 2) chaperone-mediated autophagy, and 3) macrpaotphagy, which all use the lysosome to break down cytosolic material, although via different and distinct pathways to deliver cargo. Macrpaotphagy is the most prevalent form and will hereafter be referred to as “autophagy.” The process involves specific autophagy-related (ATG) proteins that are essential for autophagy initiation and responsible for the formation of autophagosomes (5). These structures engulf entire portions of the cytosol, including ubiquitinated protein aggregates, lipid droplets, and complete organelles. Eventually, the autophagosome will fuse with a lysosomically resident lysosome to form an autolysosome, which allows degradation of the encapsulated cargo by lysosomal enzymes. Because the breakdown of cargo results in the recycling of fatty acids and amino acids for synthesis of macromolecules and also leads to energy under the form of ATP, autophagy is considered to be a beneficial process for the cell (35). Defective autophagy has been related to several age-related human disorders, including cancer, neurodegeneration, heart failure, inflammatory disorders, and atherosclerosis (3, 4, 4a, 4b, 25, 27, 31, 44). Previous reports (19, 20) have also shown that impaired autophagy in aging mice leads to increased arterial stiffness, which could be reversed by the autophagy inducer spermidine. Autophagy induction by caloric restriction or sirtuins promotes longevity and has been strongly associated with cardioprotection and antiaging effects (23, 36, 43).

Although the autophagic process has gained a lot of interest in recent years to understand its role in normal physiology (38, 47), little is known about the importance of the process in the vasculature, particularly in vascular smooth muscle (SM) cells (VSMCs). Still, autophagy in VSMCs is considered a protective mechanism against many pathophysiological stimuli, including ROS, oxidized low-density lipoproteins, and oxysterols (4a, 26, 41). Moreover, given that the induction of autophagy in VSMCs with platelet-derived growth factor (PDGF) promotes downregulation of the contractile proteins calponin and α-SM actin and increases the expression of the synthetic markers osteopontin and vimentin (39), autophagy is also known to regulate the VSMC phenotype. However, the role of autophagy in VSMC contractility remains poorly understood, even though a previous study (28) in skeletal muscle has shown that autophagy is crucial to retain contraction-related processes. Moreover, autophagy deficiency in cardiomyocytes leads to the development of structural abnormalities, accompanied by a severely decreased contractile capacity of the heart (14, 44). Defective autophagy may also lead to changes in endoplasmic reticulum (ER) size and alterations in Ca\(^{2+}\) flux (16, 17). Indeed, autophagy is stimulated by increased cytosolic Ca\(^{2+}\) concentrations through both AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR)-dependent and -independent pathways (7, 8, 12, 13, 21, 48). Based on previous findings, we believe that...
autophagy in VSMCs possibly regulates intracellular Ca\(^{2+}\) homeostasis and the contractile capacity of aortic segments. Therefore, the aim of the present study was to investigate whether a defect in VSMC autophagy has an impact on VSMC-dependent vascular reactivity and Ca\(^{2+}\) mobilization. Therefore, the aim of the present study was to investigate whether a defect in VSMC autophagy has an impact on vascular reactivity and Ca\(^{2+}\) mobilization.

MATERIALS AND METHODS

Mice. A mouse model with defective autophagy in VSMCs was developed by cross-breeding mice homozygous for the Atg7\(^{fl}\) allele (Atg7\(^{fl}\)) (18) with mice expressing Cre recombinase under control of the actin-binding transgelin (SM22alpha) promoter (SM22alpha-Cre, stock no. 004746, Jackson Laboratory). Wild-type littermates lacking the Atg7 floxed allele (Atg7\(^{+/+}\)) but expressing SM22alpha-Cre were used as a control group. The genotype of Atg7\(^{fl}\)/SM22alpha-Cre\(^{+}\) and Atg7\(^{+/+}\)/SM22alpha-Cre\(^{+}\) mice was confirmed by PCR analysis using Cre- and Atg7-specific primers (Cre: forward primer 5'-TTTGCTGCAT-TACCGGTCAAGC-3' and reverse primer 5'-TGCCCTGT- GTTCCATATCCAGGTTACGGA-3'; wild-type Atg7: forward primer 5'-CAGGACAGAGACCATCGTCCAC-3' and reverse primer 5'-GATCTTCATAAGGTGCTAGAACATGCAGG-3'; and Atg7 Flox: forward primer 5'-CAGGACAGAGACCATCAGTCCAC-3' and reverse primer 5'-TGCTGCTACTTCTGCAAT-GATGT-3'). Both male and female mice were kept in a 12:12 light-dark cycle and had access to normal chow and water ad libitum. All experiments were performed when mice reached the age of 3.5 mo, and breeding couples were not used for >5 mo. Alterations in viability or offspring segregation (Atg7\(^{+/+}\):Atg7\(^{fl}\):Atg7\(^{fl}\) = 28%:48%:24%) and differences in phenotype were not observed up to an age of 5 mo. After mice were euthanized with pentobarbital sodium (250 mg/kg ip, Nembutal, Ceva Sante Animale, Brussels, Belgium), the thoracic aorta was carefully excised and stripped from adherent tissue. Starting at the diaphragm, the descending thoracic aorta was cut in segments of 2 mm long (5–6 segments). All experiments were approved by the Ethical Committee of the University of Antwerp (2011–12).

Vascular reactivity experiments. Segments were mounted in organ chambers (10 mL) and immersed in Krebs-Ringer (KR) solution [containing (in mM) 118 NaCl, 4.7 KCl, 2.5 CaCl\(_2\), 1.2 KH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 25 NaHCO\(_3\), 0.025 CaEDTA, and 11.1 glucose; pH 7.4] at 37°C and set at 80 mm Hg of pressure and 37°C of temperature. After 1 h of stabilization, the aortic segments were exposed to a preconstricting dose of 10\(^{-4}\)M phenylephrine (PE) and then passively dilated by adding 10\(^{-5}\)M isoprenaline (ISO).

To this end, a mouse strain was developed that lacks ATG7 (Atg7\(^{fl}\)) (2011-42).

Fig. 1. Autophagy regulation and contractile apparatus in endothelium-denuded aortas and isolated vascular smooth muscle cells (VSMCs) from mice with SM cell-specific deletion of autophagy-related 7 (Atg7\(^{fl}\)). A: Western blot analysis of the autophagy-specific markers ATG7, p62, light chain 3 (LC3)-I, and LC3-II in denuded aortic segments and isolated VSMCs of Atg7\(^{+/+}\) and Atg7\(^{fl}\) mice. B: flow cytometric analysis of starved and nonstarved VSMCs stained with Cyto-ID green to determine autophagosome formation. Atg7\(^{+/+}\) and Atg7\(^{fl}\) VSMCs were starved for 4 h in PBS or treated with serum-supplemented medium (control) and then incubated with Cyto-ID for 30 min to label formed autophagosomes. Median fluorescent intensity was measured by flow cytometry, and autophagosome formation was expressed as the difference between median fluorescent intensity under starvation and control conditions. C: Western blot analysis of the contractile proteins SM myosin heavy chain (MHC), α-SM actin, and calponin in denuded aortic segments with quantification relatively expressed to β-actin. *P < 0.05 by one-sample t-test with 0 as a reference value in B and an unpaired Student’s t-test in C.

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37°C and continuously aerated with 95% O₂-5% CO₂. Aortic segments were gradually stretched and set at a preload of 16 mN for optimal force development. Tension was measured isometrically with a Statham UC2 force transducer (Gould, Cleveland, OH) connected to a Powerlab 8/30 data-acquisition system (AD Instruments, Spechbach, Germany). Isometric force was expressed in milliNewtons. Endothelium-derived relaxation by nitric oxide (NO) was prevented by inhibiting basal NO formation with 300 μM Nω-nitro-l-arginine methyl ester (l-NAME; Sigma-Aldrich). To avoid any vasomotor interference due to prostanoids, experiments were done in the presence of the cyclooxygenase inhibitor indomethacine (Certa). Contractions of aortic segments were elicited by increased concentrations of the α₁-adrenergic receptor agonist phenylephrine (PE; 10⁻⁹–10⁻⁵ M in half-log steps, Sigma-Aldrich). The different K⁺ solutions were prepared by replacing NaCl in the KR solution with equimolar amounts of KCl. Relaxation was induced with 35 μM diltiazem (Tocris) after precontraction of the segments with 50 mM K⁺. To avoid interference due to prostanoids, experiments were done in the presence of indomethacine (Certa). Contraction of 50 mM K⁺, stimulating voltage-gated Ca²⁺ channels (VGCCs) with 100 nM BAY K8644 (Tocris), or by increased concentrations of the α₁-adrenergic receptor agonist phenylephrine (PE; 10⁻⁹–10⁻⁵ M in half-log steps, Sigma-Aldrich). The different K⁺ solutions were prepared by replacing NaCl in the KR solution with equimolar amounts of KCl. Relaxation was induced with 35 μM diltiazem (Tocris) after precontraction of the segments with 50 mM K⁺ or 100 mM BAY K8644. Transient inositol 1,4,5-trisphosphate (IP₃)-mediated contractions were induced by 1 μM PE after segments were incubated for 3 min in a Ca²⁺-free environment (0Ca²⁺) under control conditions or in the presence of BAY K8644 or diltiazem. An incubation period of 3 min in 0Ca²⁺ conditions was chosen to avoid spontaneous emptying of the sarcoplasmic reticulum (SR). 0Ca²⁺ solution was prepared by omitting Ca²⁺ from the KR solution and adding 1 mM EGTA (Sigma-Aldrich) to chelate the remaining Ca²⁺ residues. To restore normal conditions hereafter, 3.5 mM Ca²⁺ was added to the 0Ca²⁺ solution. The K⁺ channel opener levocromakalim (Tocris) was used to induce membrane hyperpolarization (6).

Ca²⁺ measurements. An aortic segment (1.5 mm in length) was mounted in a wire myograph (Danish Myo Technology, Gainsville, FL) above an inverted microscope (Carl Zeiss, Zaventem, Belgium). To avoid endothelial Ca²⁺ signals, the endothelium was removed by rubbing the interior of the segment with a braided silk wax. The segment was loaded for 2 h with 10 μM fura-2 AM (Teflabs) in KR solution supplemented with 1 mg/ml BSA (Sigma-Aldrich) and 0.02% Pluronic F-127 (Sigma-Aldrich) at room temperature while it was aerated with 95% O₂-5% CO₂. Subsequently, the temperature was raised to 37°C, and the segment was set to its optimal preload. The emission (510 nm) ratio at dual excitation (340 and 380 nm) was used as a relative measure of free intracellular Ca²⁺ (in relative units) after subtraction of background emission values, which were determined by adding 2 mM MnCl₂ (Sigma-Aldrich) at the end of each experiment.

Western blot analysis. After removal of the endothelium, distal segments of the thoracic aorta and abdominal part of the aorta were lysed in Laemmli sample buffer (Bio-Rad Laboratories) containing 5% β-mercaptoethanol (Sigma-Aldrich). Samples were heated in boiling water and loaded on a Bolt 12% or 4–12% bis-Tris Plus gel (Invitrogen). After electrophoresis, proteins were transferred to Immobilon-P transfer membranes (Millipore) according to standard procedures. Membranes were blocked in Tris-buffered saline containing 5% nonfat dry milk (Bio-Rad Laboratories) for 1 h. After being blocked, membranes were probed overnight at 4°C with primary antibodies in Tris-buffered saline containing 0.05% Tween 20 (Sigma-Aldrich) and 1% nonfat dry milk followed by incubation with secondary antibodies diluted in the same buffer. After washing, membranes were rinsed and reacted with either the Signal West Pico or SuperSignal West Femto Maximum Sensitivity (Sigma-Aldrich) and 1% nonfat dry milk followed by incubation with secondary antibodies diluted in the same buffer to obtain a clearly visible band of the protein of interest (7). Membrane proteins were visualized using a BioMax-MR film (Kodak, Rochester, NY) and a BAS-2500 image analyzer (Raytest, Straubenhardt, Germany). The following primary antibodies were used: mouse anti-β-actin (clone AC-15, A5441), mouse anti-α-SM actin (clone 1A4, A2547), rabbit anti-sequestosome-1/Atg7 (A2856), rabbit anti-calpainin (ab46794), rabbit anti-sarco(endo)plasmic reticulum Ca²⁺-ATPase 2 (SERCA2; ab91032), rabbit anti-stromal interaction molecule 1 (STIM1; ab106531), rabbit anti-Orai1 (ab59330), and rabbit anti-calponin (ab46794). The following primary antibodies were used: mouse anti-β-actin (clone AC-15, A5441), mouse anti-α-SM actin (clone 1A4, A2547), rabbit anti-sequestosome-1/Atg7 (A2856), rabbit anti-calpainin (C4606), and rabbit anti-ATG7 (A2856) from Sigma-Aldrich; rabbit anti-calponin (ab46794), rabbit anti-sarco(endo)plasmic reticulum Ca²⁺-ATPase 2 (SERCA2; ab91032), rabbit anti-stromal interaction molecule 1 (STIM1; ab106531), rabbit anti-Orai1 (ab59330), and rabbit anti-calponin (ab46794). The following primary antibodies were used: mouse anti-β-actin (clone AC-15, A5441), mouse anti-α-SM actin (clone 1A4, A2547), rabbit anti-sequestosome-1/Atg7 (A2856), rabbit anti-calpainin (C4606), and rabbit anti-ATG7 (A2856) from Sigma-Aldrich; rabbit anti-calponin (ab46794), rabbit anti-sarco(endo)plasmic reticulum Ca²⁺-ATPase 2 (SERCA2; ab91032), rabbit anti-stromal interaction molecule 1 (STIM1; ab106531), rabbit anti-Orai1 (ab59330), and rabbit anti-calponin (ab46794).

![Defective autophagy in VSMCs triggers depolarization-dependent voltage-gated Ca²⁺ channel (VGCC) sensitivity and activity. A: relative concentration-response curves of contractions elicited by K⁺ in aortic segments of Atg7fl/flCre⁺ and Atg7fl/flCre⁺ mice under control conditions and in the presence of levecro-makalim. B: log EC₅₀ values of the experiments in A. C: VGCC-mediated force after the addition of 50 mM K⁺ or 100 mM BAY K8644. n = 5/group. *P < 0.05, **P < 0.01, and ***P < 0.001 by two-way ANOVA with a Bonferroni post hoc test.](http://ajpheart.physiology.org/10.1152/ajpheart.00659.2014)
rabbit anti-L-type Ca\(^{2+}\) channel α1c-subunit (Ca,1.2; ab81095) from Abcam (Cambridge, UK); mouse anti-light chain (LC)3B (0231-100B) from Nanotools (Teningen, Germany); goat anti-78-kDa glucose-regulated protein/immunoglobulin-binding protein (Grp78/BiP; SC1050) and rabbit anti-C/EBP homologous protein (sc575) from Santa Cruz Biotechnology (Dallas, TX); rabbit anti-protein disulfide isomerase (no. 3501), rabbit anti-activating transcription factor 4 (no. 9721) from Cell Signaling (Danvers, MA); and rabbit anti-plasma membrane Ca\(^{2+}\) channel \(\alpha_{1C}\)-subunit (Cav1.2; ab81095) from Abcam (Cambridge, UK) supplemented with 20% heat-inactivated FBS (Sigma-Aldrich), 100 U/ml penicillin-100 μg/ml streptomycin (Life Technologies), and 20 U/ml polymyxin B (Fagron) and allowed to attach overnight at 37°C in 95% O\(_2\)–5% CO\(_2\).

**Labeling of autophagosomes.** Freshly isolated \(\text{Atg}^7^{+/+}\) SM22α-Cre\(^+\) and \(\text{Atg}^{700}\) SM22α-Cre\(^+\) VSMCs were allowed to adhere overnight at 37°C in 95% O\(_2\)–5% CO\(_2\). Next, cells were starved in Dulbecco’s PBS (DPBS; Gibco) or treated with serum-supplemented DMEM-F-12 for 4 h. After treatment, VSMCs were washed twice with DPBS and incubated for 30 min with 1:1,000 Cyto-ID assay buffer and indicator-free RPMI (GIBCO) containing 5% FBS. Cells were then trypsinized and spun down, and the obtained cell pellet was resuspended in DPBS supplemented with 0.1% BSA and 0.05% NaN\(_3\) (Merck). Samples were analyzed by flow cytometry using a BD Accuri C6 (BD Biosciences, San Jose, CA). Unstained \(\text{Atg}^7^{+/+}\)/SM22α-Cre\(^+\) and \(\text{Atg}^{700}\)/SM22α-Cre\(^+\) VSMCs were measured in parallel to determine cell autofluorescence. The median fluorescence intensity (MFI) was used as a parameter to evaluate autophagosome formation and expressed as the difference between MFI under starvation and control conditions (∆MFI).

**X-box-binding protein 1 mRNA splicing.** Total RNA was isolated from cultured cells using the Absolutely RNA Microprep kit (Stratagene, La Jolla, CA). Alternative splicing of X-box-binding protein (XBP1) mRNA was examined by RT-PCR using XBP1-specific primers (forward primer: 5′-GATCCTGACGAGGTTCCAGAG-3′ and reverse primer: 5′-GATCCAGAAGTCCTGAG-3′) and reverse primer: 5′-GAGTCCAGATCCATGGGAA-3′.
revealed maximal contraction responses. For each vessel segment, were fitted with sigmoidal equations with variable slope, which presents the number of mice. Relative concentration-response curves of the contractile component, Y = 0 is the start amplitude (in this case, 0 mN), A_{contraction} is the amplitude of the contractile component, K_{contraction} is the rate constant of the contractile component, A_{relaxation} is the amplitude of the relaxing component, K_{relaxation} is the rate constant of the relaxing component, and X0 is the time at which force initiates. The time constant (τ) was calculated as 1/K_{contraction} for the contractile component and 1/K_{relaxation} for the relaxing component of the IP3-mediated contraction. Two-way ANOVA with a Bonferroni multiple-comparison post hoc test and unpaired Student’s t-test were used to compare means of the different experimental groups (GraphPad Prism, version 5, GraphPad Software, San Diego, CA, and SPSS 22.0, IBM, Armonk, NY). A 5% level of significance was selected.

RESULTS

Autophagy is defective in VSMCs of Atg7−/− SM22α-Cre+ mice. The essential autophagy gene Atg7 was excised in VSMCs by crossbreeding mice homozygous for Atg7−/− with a transgenic mouse strain that expresses Cre recombinase under control of the SM22α promoter. Western blot analysis of endothelium-denuded aortas and freshly isolated VSMCs showed elimination of ATG7 in VSMCs of Atg7−/− SM22α-Cre+ mice (Fig. 1A). Typical features of impaired autophagy, such as the accumulation of sequestosome-1/p62 and prevention of microtubule-associated protein 1 LC3 processing (Fig. 1A), were observed. Moreover, autophagosome formation was inhibited in VSMCs of Atg7−/− SM22α-Cre+ mice under starvation conditions, indicating autophagy deficiency (Fig. 1B).

Expression of the contractile proteins SM myosin heavy chain, α-SM actin, and calponin was not altered in Atg7−/− SM22α-Cre+ mice (Fig. 1C).

Defective autophagy in VSMCs increases VGCC expression and triggers VGCC sensitivity and activity. Depolarization-mediated contractions of Atg7−/− SM22α-Cre+ aortic segments reached their maximum at significantly lower K+ concentrations compared with aortic segments of Atg7+/+ SM22α-Cre+ mice. In the presence of the hyperpolarizing agent levromakalin, K+ concentration-response curves shifted equally to the right and remained significantly different (Fig. 2A). Corresponding log EC50 values of the concentration-contraction curves confirmed the increased sensitivity of VGCCs in Atg7−/− SM22α-Cre+ mice under normal and levromakalin treatment.
conditions (Fig. 2B). Moreover, significantly higher contractions were observed in Atg7fl/ fl SM22α-Cre+ segments after depolarization with 50 mM K+ or stimulation with 100 nM BAY K8644 under basal conditions (Fig. 2C). These effects could be completely inhibited with 35 μM diltiazem, indicating that the contractions were entirely VGCC mediated (34). Higher contractions were the result of increased VGCC-mediated Ca2+ influx, as demonstrated by the depolarization of segments with 50 mM K+ (Fig. 3A). In line with these findings, Western blot experiments showed increased expression of Cav1.2, which forms the pore of the VGCC (Fig. 3B). Concentration-response curves with PE were not significantly different, indicating that α1-receptor sensitivity in Atg7fl/ fl SM22α-Cre+ mice was unaltered (Fig. 4, A and B). Furthermore, no significant differences in absolute force were observed after contraction of segments with 3 × 10−6 M PE (Atg7fl/ fl SM22α-Cre+ vs. Atg7+/+ SM22α-Cre+ segments: 12.2 ± 0.5 vs. 11.3 ± 0.4 mN, P > 0.05).

Fig. 6. Defective autophagy in VSMCs increases the importance of VGCCs in refilling the SR under basal conditions. A and B: IP3-mediated transient contractions of Atg7+/+ Cre+ and Atg7fl/ fl Cre+ aortic segments and changes in area under the curve (ΔAUC) of the respective contractions after preincubation with 35 μM diltiazem (A) or 100 nM BAY K8644 (B) for 10 min. C: absolute AUCs of IP3-mediated contractions in control conditions and in the presence of 100 nM BAY K8644. D: Western blot analysis of Ca2+ release-activated channel (CRAC)-mediated proteins stromal interaction molecule 1 (STIM1) and Orai1. n = 5/group in A–C and 4/group in D. *P < 0.05, **P < 0.01, and ***P < 0.001 by two-way ANOVA with a Bonferroni post hoc test and unpaired Student’s t-test for ΔAUC.
Defective autophagy in VSMCs promotes IP₃-mediated contractions and alters Ca²⁺ homeostasis. IP₃-mediated contractions of Atg7fl SM22α-Cre⁺ segments were significantly higher compared with those of Atg7fl⁺/+ SM22α-Cre⁺ segments (Fig. 5A). Both the contractile component and relaxing component developed slower in Atg7fl⁻/⁻ SM22α-Cre⁺ segments (τ of the contractile component: 5.3 ± 0.6 s vs. 2.9 ± 0.2 s, *P* < 0.01; and τ of the relaxing component: 25.4 ± 3.4 s vs. 13.5 ± 1.7 s, *P* < 0.05). Moreover, PMCA expression was decreased in VSMCs of Atg7fl⁻/⁻ SM22α-Cre⁺ mice (Fig. 5B), hampering the extrusion of Ca²⁺ from the cytosol to the extracellular environment. Interestingly, Atg7fl⁻/⁻ SM22α-Cre⁺ VSMCs showed increased expression of SERCA2 (Fig. 5B), which pumps Ca²⁺ from the cytosol into the SR. The spontaneous release of Ca²⁺ from the SR into the cytosol was reduced in Atg7fl⁻/⁻ SM22α-Cre⁺ VSMCs, as the time-dependent decrease of IP₃-mediated contractions was significantly lower after incubation of segments for 3, 5, 10, and 15 min in 0Ca²⁺ solution (data not shown). Blockade of VGCCs by preincubation of aortic segments with 35 μM diltiazem lowered IP₃-mediated contractions more effectively in Atg7fl⁻/⁻ SM22α-Cre⁺ mice (Fig. 6A). However, stimulation of VGCCs by preincubation of aortic segments with 100 nM BAY K8644 led to increases in IP₃-mediated contractions that were equal for both groups (Fig. 6B) and were only significantly different in Atg7fl⁻/⁻ SM22α-Cre⁺ mice compared with control conditions (Fig. 6C). Basal tonus did not completely normalize in the presence of BAY K8644 under 0Ca²⁺ conditions and was significantly higher in aortic segments of Atg7fl⁻/⁻ SM22α-Cre⁺ mice (Fig. 6B). Expression of Orai1 and STIM1, which are both essential for the activation of Ca²⁺ release-activated channels (CRACs), was unaltered (Fig. 6D). Under basal conditions, VSMCs of Atg7fl⁻/⁻ SM22α-Cre⁺ mice showed increased cytosolic Ca²⁺ levels (Fig. 7A). In line with this finding, removal of cytosolic Ca²⁺ by 0Ca²⁺ solution decreased Ca²⁺ concentrations significantly more in Atg7fl⁻/⁻ SM22α-Cre⁺ VSMCs (Fig. 7A). The release of Ca²⁺ from the SR by 10 μM cyclopiazonic acid in 0Ca²⁺ conditions induced a significantly higher relative Ca²⁺ signal in Atg7fl⁻/⁻ SM22α-
segments (Fig. 7B), indicating increased SR Ca\(^{2+}\) load. Furthermore, although no effects were seen on absolute force elicited by store-operated Ca\(^{2+}\) entry (SOCE) after the addition of 3.5 mM Ca\(^{2+}\) to the 0Ca\(^{2+}\) environment, the corresponding Ca\(^{2+}\) signal was significantly higher in VSMCs of Atg7\(^{-/-}\) SM22\(\alpha\)-Cre\(^{+}\) mice (Fig. 7C).

Defective autophagy leads to expansion of the SR without the occurrence of SR stress. Expression of the Ca\(^{2+}\)-binding SR chaperone molecules calnexin, calreticulin, Grp78/BiP, and protein disulfide isomerase was increased in Atg7\(^{-/-}\) SM22\(\alpha\)-Cre\(^{+}\) VSMCs (Fig. 8). Although Grp78/BiP expression was upregulated in Atg7\(^{-/-}\) SM22\(\alpha\)-Cre\(^{+}\) VSMCs, XBP1 splicing and expression of the ER stress markers phospho-eIF2\(\alpha\), activating transcription factor 4, and C/EBP homologous protein were not elevated, indicating the absence of SR stress (Fig. 9, A and B). Aortic VSMCs of Atg7\(^{-/-}\) SM22\(\alpha\)-Cre\(^{+}\) and Atg7\(^{-/-}\) SM22\(\alpha\)-Cre\(^{+}\) mice were isolated, and the SR was stained with an ER/SR tracker. Fluorescence microscopy and flow cytometry experiments showed that the fluorescence intensity of the SR was significantly higher in VSMCS of Atg7\(^{-/-}\) SM22\(\alpha\)-Cre\(^{+}\) mice (Fig. 10, A and B).

**DISCUSSION**

VSMCs have an important regulatory function in the cardiovascular system as they determine the contractile and relaxing properties of blood vessels. The goal of the present study was to clarify the role of autophagy in VSMCs by investigating its effect on vascular contractility and Ca\(^{2+}\) homeostasis. Our results indicate that the contractile capacity and Ca\(^{2+}\) mobilization in VSMCs are dependent on autophagy and that loss of this process leads to changes in Ca\(^{2+}\) homeo-
stasis and enhanced vascular reactivity, independent of alterations in the contractile apparatus. A schematic overview of the differences between $\text{Atg7}^{+/+}$ SM22α-Cre$^+$ and $\text{Atg7}^{+/+}$ SM22α-Cre$^+$ VSMCs is shown in Fig. 11. First, we demonstrate that defective autophagy in VSMCs triggers VGCC expression, resulting in higher depolarization- and agonist-mediated contractions. VGCCs of $\text{Atg7}^{+/+}$ SM22α-Cre$^+$ VSMCs are also more sensitive to depolarization, which is independent of the altered VSMC resting membrane potential as $K^+$ concentration-response curves of $\text{Atg7}^{+/+}$ SM22α-Cre$^+$ and $\text{Atg7}^{+/+}$ SM22α-Cre$^+$ aortic segments remained significantly different under levcromakalim treatment conditions. Levcromakalim hyperpolarizes the membrane and sets the resting potential on a fixed value of $\pm$ −80 mV, thereby preventing changes in $K^+$ sensitivity that result from differences in membrane potential. To our knowledge, this is the first evidence that autophagy alters VSMC contraction through increased expression of VGCCs with higher depolarization sensitivity.

Second, autophagy-defective VSMCs display an expanded SR with an increased Ca$^{2+}$ storing capacity, resulting in significantly higher IP$_3$-mediated contractions upon Ca$^{2+}$ release with PE. Recent evidence shows that autophagy regulates cellular homeostasis by selectively degrading organelles or parts of organelles (37). Because ER constituents are present in larger proportions than cytoplasmic proteins in autophagosomes, it can be assumed that the ER is selectively targeted to prevent further ER biogenesis (9). Therefore, our findings indicate that autophagy in VSMCs exhibits a regulatory function in controlling cellular homeostasis, which directly affects Ca$^{2+}$ storage and indirectly influences contractions elicited upon $\alpha_1$-receptor stimulation. The amount of Ca$^{2+}$ stored in

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**Fig. 11.** Overview of the effects of defective autophagy in VSMCs on contractility and Ca$^{2+}$ mobilization. A and B: differences between $\text{Atg7}^{+/+}$ Cre$^+$ (A) and $\text{Atg7}^{+/+}$ Cre$^+$ (B) VSMCs. Defective autophagy in VSMCs causes an imbalance between Ca$^{2+}$ release/influx and Ca$^{2+}$ reuptake/extrusion through 1) increased VGCC expression and activity, 2) expansion of the SR, 3) increased SERCA2 expression, 4) higher store-operated Ca$^{2+}$ entry (SOCE), and 5) decreased PMCA expression, which result in higher IP$_3$-mediated contractions, depolarization-mediated contractions, and elevated basal Ca$^{2+}$ levels. PLC, phospholipase C.
VGCC activity promotes SOCE-mediated Ca\textsuperscript{2+} autophagy-defective VSMCs. Our data indicate that increased Orai1 needs to link with oligomerized STIM1 molecules at the VGCCs, the CRAC pore-forming plasma membrane protein was not evident in chaperone molecule Grp78/Bip, the occurrence of SR stress splicing of XBP1 was not elevated in these cells. Moreover, the inhibition of the UPR was absent in turn, might trigger the unfolded protein response (UPR) and increased chaperone assistance is required to prevent the load of the SR.

In cells with an expanded ER, such as plasma cells and pancreatic β-cells, protein trafficking is considerably higher and increased chaperone assistance is required to prevent the accumulation of partially folded or misfolded proteins in the ER (22). In addition, defective autophagy, as such, could result in the accumulation of misfolded proteins in the SR, which, in turn, might trigger the unfolded protein response (UPR) and production of chaperone molecules (50). However, the initiation of the UPR was absent in Atg7\textsuperscript{-/-} SM22α-Cre\textsuperscript{+} VSMCs, as splicing of XBP1 was not elevated in these cells. Moreover, despite the increased expression of the SR stress marker and chaperone molecule Grp78/Bip, the occurrence of SR stress was not evident in Atg7\textsuperscript{-/-} SM22α-Cre\textsuperscript{+} VSMCs. Therefore, we assume that the upregulation of SR chaperone molecules in VSMCs of Atg7\textsuperscript{-/-} SM22α-Cre\textsuperscript{+} mice is merely originating from an enlarged SR and that their capacity of coping with unfolded proteins is not surpassed, thereby preventing SR stress. Interestingly, expansion of the ER in ATG7-deficient T lymphocytes is associated with reduced SOCE-mediated influx due to impaired STIM1 redistribution (16, 17). This feature was not observed in Atg7\textsuperscript{-/-} SM22α-Cre\textsuperscript{+} VSMCs. Apart from VGCCs, the CRAC pore-forming plasma membrane protein Orai1 needs to link with oligomerized STIM1 molecules at the SR membrane to initiate SOCE (1, 28, 31, 34). In the present study, we showed that STIM1-Orai1 signaling was intact in autophagy-defective VSMCs. Our data indicate that increased VGCC activity promotes SOCE-mediated Ca\textsuperscript{2+} influx in Atg7\textsuperscript{-/-} SM22α-Cre\textsuperscript{-} VSMCs, which, together with higher SERCA2, increases Ca\textsuperscript{2+} storage in the expanded SR and that decreased PMCA protein expression in combination with a fully loaded SR raises basal cytosolic Ca\textsuperscript{2+} concentrations.

It should be noted that our results cannot be extrapolated to other types of muscle cells. Taneike et al. (44) demonstrated that cardiomyocyte-specific deletion of Atg5 leads to decreased contractile function of the heart. Moreover, Masiero et al. (29) showed that Atg7-specific deletion in skeletal muscle tissue resulted in muscle loss and lowered muscular force production, which is in line with in vivo observations showing that inhibition of autophagy in skeletal muscle cells can lead to severe myopathies, such as Danon disease and Pompe disease (24, 28, 29). Therefore, we can state that autophagy affects contraction differently, dependent on the muscle type.

In conclusion, we provide novel insights in the role of autophagy in VSMC contractility and Ca\textsuperscript{2+} homeostasis, thereby further specifying its function in maintaining normal physiology and its influence on cellular processes that are important in the cardiovascular system.

**ACKNOWLEDGMENTS**

The authors thank Dr. Masaki Komatsu (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) for providing Atg7\textsuperscript{-/-} mice.

**GRANTS**

This work was funded by the Fund for Scientific Research-Flanders and the University of Antwerp. C. F. Michiels is a fellow of the Agency for Innovation through Science and Technology.

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